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Carbonylation of cornified envelopes in the stratum corneum

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Abstract Stratum corneum (SC), the outermost layer of the skin, is continuously exposed to oxidative stress via sunlight, lipid peroxidation, and is subsequently accompanied by oxidative modification. Previous studies have shown that major oxidative target proteins in the SC are keratins. However, it remains unclear to date whether cornified envelopes (CEs), protein envelopes of the corneocytes (cornified cells), would be oxidized. In this study, we first revealed oxidative modification of CEs using labeled hydrazide derivatives to detect carbonyl moieties. Carbonylation of CEs was confirmed by reaction with monoclonal antibodies against aldehyde-bound proteins, including anti-acrolein, anti-crotonaldehyde, anti-4-hydroxy-2-nonenal. The extent of carbonylation is stronger in CEs from the face, a sun-exposed area, than those from the inside of upper arm, an unexposed area. Carbonylation of CEs did not depend on their maturity, as evaluated by loss of involucrin antigenicity during maturation process, suggesting that CEs are carbonylated regardless of their maturation stage.

Keywords: Cornified envelope; Stratum corneum; Carbonyl; Aldehyde

1. Introduction

The skin is an interface of body and environment, and is continuously exposed to external stimuli, including oxidative stress, dry environment, chemical hazards, microorganisms, and so on. Reactive oxygen species, induced by oxidative stress, can immediately attack the constituents of the skin and may affect functions of the skin. One of the susceptible molecules of oxidative stress is a group of skin surface lipids, especially sebum-derived squalene and unsaturated fatty acids. Consequent lipid peroxides are very reactive and harmful to the cutaneous cells [1].

It is well established that proteins are also targets for oxidative stress [2–5]. Certain amino acid side residues, such as lysine, histidine, tyrosine, cysteine, and so on, are susceptible to oxidative stress, leading to structural or conformational changes, which are often associated with altered function of the proteins; for instance, loss of catalytic activity in the enzymes [2,3]. Protein carbonyls may be formed either by oxidative cleavage of proteins, or by direct oxidation of amino acid residues. In addition, carbonyl groups may be introduced into proteins via reaction with aldehydes derived from lipid peroxidation. The presence of carbonylated protein, which can be detected by labeling with 2,4-dinitrophenylhydrazide, has been widely used as a marker of reactive oxygen-mediated alteration of proteins, which is often associated with aging and some disease [2–5].

It has been revealed that carbonylated proteins can also be detected in the skin, especially increased level in the dermis of photo-damaged skin and solar elastosis [6,7]. In addition to dermis, the stratum corneum (SC), which is the outermost layer of the epidermis and is exposed severely to oxidative stress, has shown to be carbonylated. Thiele and his colleagues [8–11] extensively studied on protein oxidation in the SC and revealed that higher levels of carbonyls were detected in the upper layer of SC of sun-exposed area, and that major susceptible protein to oxidative stress is keratins, shown by a Western blotting technique [8].

SC is a composite architecture, consisted of corneocytes and intercellular lipids, and plays a pivotal role in permeability barrier function of the skin. Cornified envelope (CE) is a thin insoluble structure surrounding corneocytes, and is formed via complex but well-organized processes [12,13]. During terminal differentiation of epidermal keratinocytes, CE precursor proteins, including involucrin, loricrin, and others, are expressed and crosslinked via the formation of γ-glutamyl-ε-lysine isopeptide bonds by Ca^{2+}-dependent transglutaminases (EC 2.3.2.13) [12–14], which catalyze transfer of γ-carboxy group of glutamine in the protein to primary amines including ε-amino group of peptidyl lysine. Exterior surface of CEs was esterified with ω-hydroxyceramides, and onto this hydrophobic assembly lamellar structure of intercellular lipids are organized and play an essential role in barrier function of the skin [13].

We have previously established a method to evaluate CE maturity by means of double staining with anti-involucirn antibody and Nile red, which is based on loss of involucrin antigenicity and acquisition of hydrophobicity during maturation of CE in the SC [15,16], and have shown that immature CEs were abundantly detected in the deeper layer of SC, as well as the SC with poor barrier function, such as the face [15], inflammatory disorders [16], and scars [17].

Although CEs are consisted of crosslinked proteins and are assumed to be susceptible to carbonylation as well as keratins, it has not been elucidated whether CEs are carbonylated or not. In this study, we demonstrated carbonylation of CEs and compared them between sun-exposed area and unexposed area.

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Abbreviations: BSA, bovine serum albumin; CE, cornified envelope; PBS, Dulbecco's phosphate-buffered saline; SC, stratum corneum

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2. Materials and methods

2.1. SC samples

Samples were collected from the outermost SC of the cheek or the inside of upper arm of healthy volunteers by a non-invasive tape stripping using cellophane tape (Nichiban CT-24, Tokyo) or the equivalent adhesive tapes (e.g., D-Squame, CuDerm, Dallas, TX). Informed consents were obtained prior to collection in all cases.

2.2. Preparation of CEs from SC samples

SC samples were immersed in a dissociation buffer consisting of 2% sodium dodecyl sulfate–20 mM dithiothreitol–5 mM EDTA–0.1 M Tris–HCl (pH 8.5), and incubated at 100 °C for 10 min, followed by centrifugation at 7000 × *g* for 10 min. Resulted supernatants consisted of soluble substances were discarded, and boiling and washing procedure of the pellet with the dissociation buffer was repeated more three times to remove soluble substances extensively. Resulted pellets of CEs were subjected for further characterization.

2.3. Detection of carbonyls in CEs

SC samples on adhesive tapes were incubated in 0.5 mM biotin–hydrazide (Molecular Probes, Eugene, OR) in 0.1 M 2-morpholinoethane sulfonic acid (MES)-Na buffer (pH 5.5) for 1 h at room temperature to label carbonyls in the SC with biotin. Thereafter, CEs were prepared from the SC samples as described above. CE suspension was spotted onto a slide glass, air-dried, and fixed in cold acetone (−20 °C, 10 min). They were stained with fluorescein-labeled or Texas Red-labeled streptavidin (1:100, Amersham Biosciences, Buckinghamshire, UK) diluted in 3% bovine serum albumin (BSA) (Sigma–Aldrich, St. Louis, MO) in Dulbecco's phosphate-buffered saline (pH 7.4) (PBS) at 4 °C overnight. Fluorescence images were obtained using a fluorescence microscope (Olympus, Tokyo) equipped with a CCD camera (SPOT, Diagnostic Instruments, MI). Filter combination of a band-path filter 460–490 nm and a dichroic mirror 505 nm are used to detect fluorescence of fluorescein, and a band-path filter 520–550 nm and a dichroic mirror 565 nm for Texas Red.

2.4. Immunochemical detection of carbonyls on CEs

A series of monoclonal antibodies against aldehyde-modified protein, including anti-acrolein, anti-crotonaldehyde, anti-4-hydroxy-2-hexenal, anti-4-hydroxy-2-nonenal, and anti-malondialdehyde, were obtained from Nippon Oil and Fat Co. (Tokyo, Japan). CEs were prepared from the SC samples and fixed onto slide glass as described above, and they were stained with anti-aldehyde antibody (1:50, diluted with 3% BSA in PBS) at 4 °C overnight followed by fluorescein-labeled anti-mouse Ig (1:100, Amersham Biosciences) diluted with 3% BSA in PBS at 25 °C for 2 h to detect aldehyde-modified proteins on CEs.

2.5. Evaluation of CE maturity by involucrin staining

Maturity of CEs was evaluated by the method described previously [15]. Briefly, CEs were prepared from the SC samples and fixed onto slide glass as described above, and they were stained with anti-involucrin (clone SY5, Novocastra, Newcastle upon Tyne, UK, 1:100, diluted with 3% BSA in PBS) at 4 °C overnight followed by fluorescein-labeled anti-mouse Ig (1:100, diluted with 3% BSA in PBS) at 25 °C for 2 h to evaluate the loss of involucrin antigenicity during maturation. Fluorescence images were obtained using a fluorescence microscope.

3. Results

3.1. Detection of carbonyls in CEs by a hydrazide derivative

Employing the well-established procedure to detect protein carbonyls with labeled hydrazide, CEs, which were obtained as insoluble substances from the SC, were shown to be decorated with carbonyl moieties (Fig. 1). Although intensity of the fluorescence is semi-quantitative, increased carbonylation was observed in CEs from the face, sun-exposed area, as compared with those from the inside of upper arm, unexposed area. CEs from the inside of upper arm were homogeneously and weakly stained in their periphery. In contrast, CEs from the face exhibited heterogeneous staining of carbonyl protein; some of the face CEs were stained strongly, but not the rest. Increased carbonyls in CEs from the face was in agreement with previous findings that SC carbonyl level in the sun-exposed area was higher than that in non-exposed area [9].

3.2. Characterization of carbonyl CEs by monoclonal antibodies against aldehyde-modified proteins

To confirm carbonylation of CEs, we examined immunochemically whether CEs are reactive with the monoclonal antibodies raised against aldehyde-modified proteins. As shown in Fig. 2, CEs from the face could be stained with anti-acrolein and anti-crotonaldehyde antibodies, and weakly stained with anti-4-hydroxy-2-hexenal and anti-4-hydroxy-2-nonenal, but not with anti-malondialdehyde. These results clearly demonstrated that carbonylation of CEs was mediated, at least in part, by reaction of CE components with some aldehydes including acrolein and crotonaldehyde.

3.3. Comparison of CE maturity and carbonylation

Heterogeneous increase in carbonylation in the face CEs led us to investigate relationship between CE maturation and carbonylation, since we previously showed that the face CEs were heterogeneous in maturity and consisted of abundant immature CEs [15].

As reported previously, involucrin-positive immature CEs were existed in the outermost layer of the face SC (Fig. 3A), but not in the inside of upper arm (Fig. 3B). Simultaneous detection of carbonyl CEs and immature CEs was successfully carried out by double staining as follows; carbonyls were labeled with biotin–hydrazide followed by Texas Red-streptavidin, whereas immature CEs were stained with anti-involucrin followed by fluorescein-labeled secondary antibody. As shown in Fig. 3C, CEs from the face SC were strikingly heterogeneous; some of them exhibited red fluorescence, showing carbonylated mature CEs, some of them exhibited green fluorescence, showing immature CEs without carbonylation, and a small part of them exhibited yellow fluorescence, showing carbonylated immature CEs. This result clearly suggested that the face CEs were consisted of carbonyl CEs and immature CEs, but they did not always coincide with each other (Fig. 3C). In contrast, most of CEs from the inside of upper arm exhibited homogeneous red fluorescence suggesting carbonylated mature CEs (Fig. 3D).

4. Discussion

In this study, we demonstrated that CEs, as well as keratins, were also the target of carbonylation. We employed two methods to detect carbonylatioin of CEs; one is detection of protein carbonyls by labeling with hydrazine derivatives, and the other is detection of aldehyde adducts with specific antibodies. A series of monoclonal antibodies raised against aldehyde-modified proteins are highly useful to demonstrate existence of modified proteins immunohistochemically [18–20]. For example, it has been successfully revealed that acrolein adduct in oxidatively modified human low density lipoprotein [19]. We also applied these antibodies to detect aldehyde adducts in the CEs, and clearly confirmed that CEs are also decorated with some

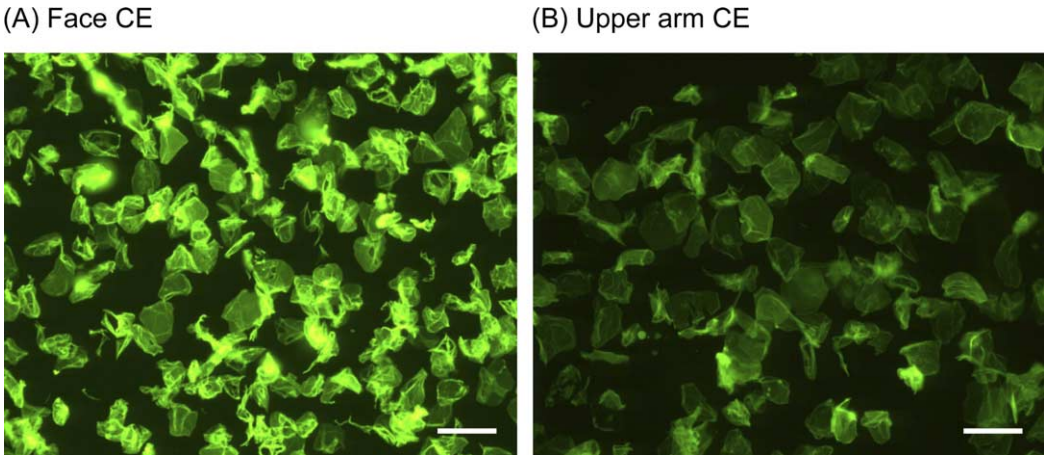

Fig. 1. Detection of carbonyls in CEs by reaction with a hydrazide derivative. Tape-stripped SC was first reacted with biotin–hydrazide to label carbonyl moieties, and then CEs were isolated from the SC, followed by reaction with fluorescein-labeled streptavidin. (A) CEs from the face SC, (B) CEs from the SC of the inside of upper arm. Bar = 50 μm. Note the highly carbonylated CEs existed in the face SC.

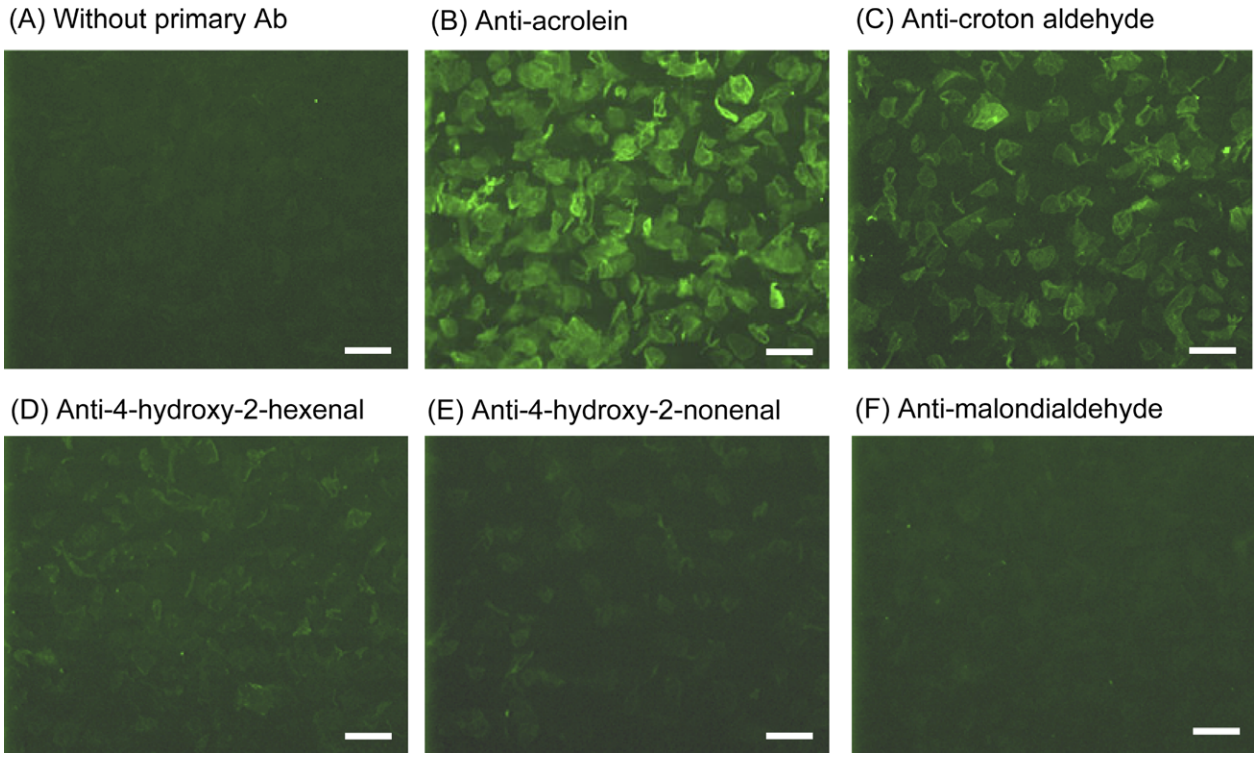

Fig. 2. Immunochemical detection of carbonyls in CEs by reaction with monoclonal antibodies against aldehyde-modified proteins. CEs were prepared from the face SC, and reacted without (A) or with a series of antibodies, anti-acrolein (B), anti-croton aldehyde (C), anti-4-hydroxy-2-hexenal (D), anti-4-hydroxy-2-nonenal (E), and anti-malondialdehyde (F), followed by fluorescein-labeled secondary antibody. Bar = 50 μm.

aldehydes, suggesting that carbonylated CEs, as detected with hydrazide derivatives, are consisted of, at least in part, those modified with aldehydes. However, since some of the aldehyde adducts may be heat labile (Dr. K. Uchida, personal communication), heat treatment during preparation of CEs may reduce or diminish the level of the aldehyde adducts recognized by the antibodies. Determination with intact samples or CEs prepared without heat treatment, both of which are associated with practical difficulties, might brought more precise information of properties of CE carbonylation.

Existence of such aldehydes on the surface of the skin suggested contribution of sebaceous lipids peroxidation to carbonylation of CEs. In addition, peroxidation may be occurred in the constituents of intercellular lipids surrounding corneocytes, that may be likely to attack CEs nearby. Furthermore, certain lipids, which are covalently attached to the exterior surface of CEs, consisting ω-hydroxy ceramides and ω-hydroxy fatty acids, have unsaturated aliphatic chains that could be oxidized and converted into carbonyl moieties. Precise chemical structures of carbonyl adducts on CEs are remained to be elucidated.

Our previous studies revealed that defect of CE maturation in the SC associated with poor barrier function, such as the face [15] and inflammatory skin disorders [16]. Increased carbonylation level in the face CEs shown in this study led

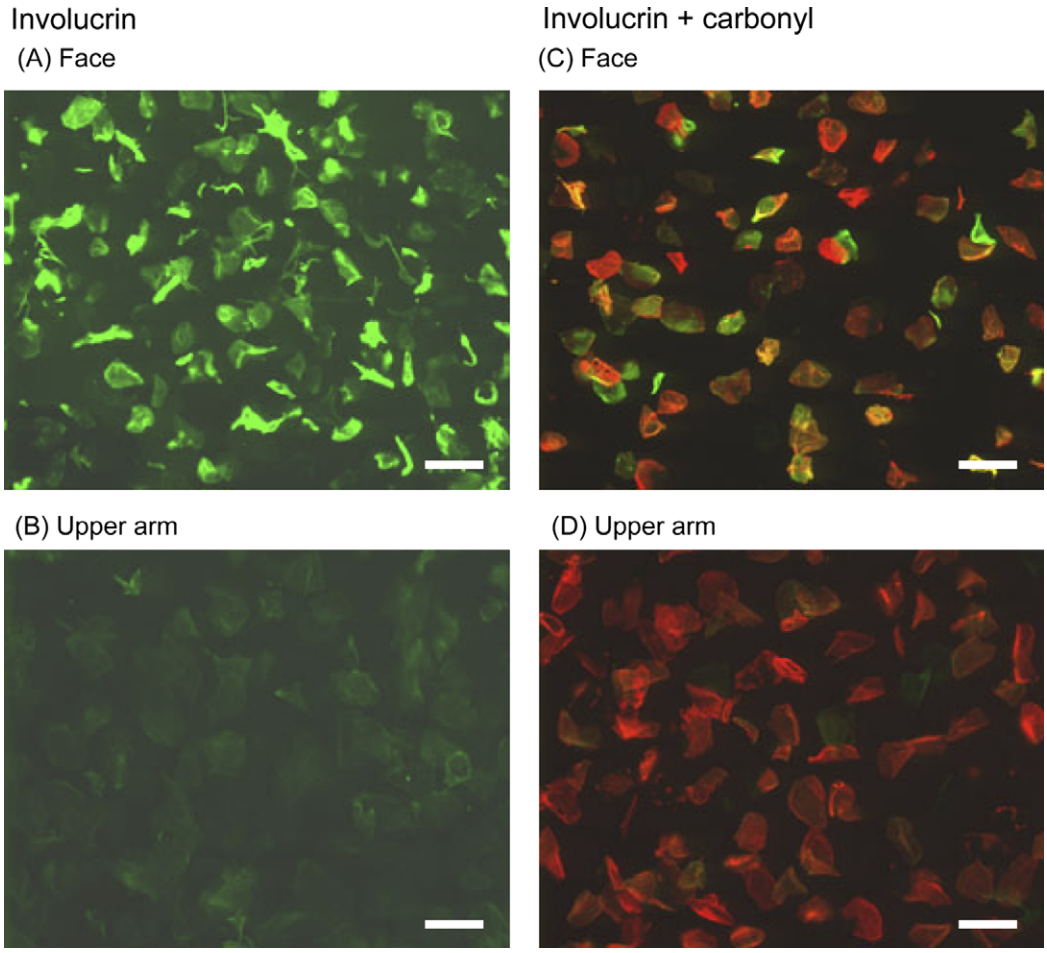

Fig. 3. Comparison of carbonylated CEs with immature CEs. Tape-stripped SC from the face was first reacted with biotin–hydrazide to label carbonyl moieties, and then CEs were isolated from the SC. Immature CEs were detected based on involucrin antigenicity by combination of anti-involucrin and fluorescein-labeled secondary antibody (A, B), while carbonylated CEs were finally detected by reaction with Texas Red-streptavidin (C, D). CEs from the face SC (A, C) and CEs from the SC (B, D) of the inside of upper arm. Bar = 50 μm. Note discrepancy between carbonylated CEs (red) and immature CEs (green) in the face CEs (C).

us to precise investigation on the relationship between carbonylation of CEs and their maturity. Although the face CEs exhibited both less maturity and more carbonyl than the inside of upper arm, immature CEs and carbonyl CEs were not identical at each cellular level (Fig. 3C). Carbonylation can modify certain amino acid residues including ε-amino group of lysine, which can also be employed for formation of γ-glutamyl-ε-lysine isopeptide bond in CE maturation. Then, we hypothesized that carbonyl modification may be inhibitory in CE maturation, and retardation of CE maturation in the sun-exposed face SC may potentially be attributable to carbonyl modification. However, the result that immature CEs and carbonyl CEs were not identical (Fig. 3C) did not support this hypothesis. Biological significance of carbonylation of CEs in the SC function remains to be elucidated.

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